Journal of Cellular Biochemistry 56:315–322 (1994)

# Understanding Bone Cell Biology Requires an Integrated Approach: Reliable Opportunities to Study Osteoclast Biology In Vivo

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**Abstract** The relative simplicity of all in vitro methods to study bone cell biology will at best result in oversimplification of the development and functional capacity of the skeleton in vivo. We have shown this to be true for selected aspects of bone cell biology, but numerous other examples are available.

One alternative is to undertake skeletal research in vivo. It is important that those in bone research be willing to move increasingly in this direction not only to understand the true complexitities of skeletal versatility, but also to avoid repetition and perpetuation of erroneous or irrelevant conclusions which waste resources.

Toward this end we have described two situations, osteopetrosis and tooth eruption, in which reproducible abrogations or local activations of bone resorption can be examined in vivo. The application of emerging molecular and morphological techniques that permit the subcellular dissection of metabolic pathways and their precise cellular localization, such as a combination of the variety of in situ hybridization technologies with PCR, antisense probes, and antibody blockase, will allow the investigator greater control of variables in vivo. We expect that these technologies, largely worked out in vitro, combined with highly selected, appropriate models, as we have ourlined here for *osteoclast* biology, will make research in vivo less intimidating and increase the frequency with which the real biology is studied directly. 

**Key words:** bone, cell biology, osteopetrosis, tooth eruption, osteoclast

In the final analysis, all biology, like politics, is local, to paraphrase the former Speaker of the U.S. House of Representatives, Thomas "Tip" O'Neill. As in most fields of biological research, bone cell biologists are confronted with the problem of how to study the complex *local* regulation of skeletal metabolism. Skeletal metabolism consists of the production and mineralization of a complex matrix by one group of cells and removal of the mineralized matrix by another. These processes are all timed and coordinated so that, in skeletal development, matrix formation and mineralization exceed resorption to produce a functional skeleton and later these processes are coordinated and balanced to maintain skeletal mass. These successive periods require a precise coordination of the development and function of several different cell populations, and may involve different mechanisms for each. In a sincere scientific effort to limit the known and unknown variables operating in vivo, bone cell biologists have used an ever increasing variety of cultured bone cell and tissue preparations to study skeletal development and regulation [Marks and Popoff, 1988]. While in vitro approaches have provided reliable observations in some instances, they have been almost universally deficient in demonstrating the mechanisms for precise, local regulation of skeletal metabolism. This is in part due to the redundancy of such controls, lessons which are emerging from the initially disappointing but surprising results from specific gene knockouts [Erickson, 1993]. On another level in vitro observations suffer from the varying inability of culture conditions to duplicate those in vivo.

Specifically, in the recent past, studies of macrophages as osteoclast surrogates [Kahn et al., 1978] and cloned osteosarcoma cells as osteoblast surrogates [Majeska and Rodan, 1982] have been disappointing at best and erroneous at

Received March 31, 1994; accepted April 1, 1994.

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worst [Hattersley and Chambers, 1989; Grigoriadis et al., 1985]. Similarly, studies of the effects of the growth factor colony-stimulating factor-1 (CSF-1) on cultured osteoclasts have been contradictory [Tanaka et al., 1993; Shinar et al., 1990]. In addition, the use of bone organ cultures in the initial report of the skeletal effects of the prostaglandins produced limited, erroneous information about their true effect in vivo [Marks and Miller, 1993]. Because organ cultures of bone invariably resorb, the initial report described resorption as the major effect [Klein and Raisz, 1970], and it has taken more than two decades to reverse this view and establish enhanced bone formation [Ueda et al., 1980; Mori et al., 1990; Miller and Marks, 1993] as the true effect in vivo. Finally, no current methods for the isolation and culture of specific bone cells can produce pure populations, and contaminants are seldom characterized with precision [Chatterjee et al., 1992]. At best, these methods permit evaluation of either bone formation or resorption in presumed isolation, a phenomenon rarely encountered in vivo.

What are the alternatives to the limitations of present culture methods? We believe there are two. The first is to rigorously verify the biological meaning of all observations from cultured cells by comparing them to what is known in vivo. This principle is well recognized by those in the biotech industry. The second is to do the experiment in vivo. While the latter is rather intimidating given all the unknowns in the real world, chances for success can be markedly improved by taking advantage of the judicious use of nature's own experiments (mutations) or focusing on precisely timed, site-specific, and predictable local manifestations of bone metabolism. We present below the rationale and resources for studying osteoclast biology in vivo using osteopetrotic mutations and tooth eruption, a local, bilaterally symmetrical metabolic event in alveolar bone. These involve a generalized compromise and a local activation of osteoclast development and function, respectively. We believe that, while we have already learned much about osteoclast biology by studying congenital osteopetrosis and tooth eruption, they have much more to reveal about bone cell biology.

## OSTEOCLAST BIOLOGY

Osteoclasts are large, multinucleated cells of bone responsible for bone resorption. The morphological hallmark of active osteoclasts is the ruffled border, a uniquely polarized region consisting of membrane foldings which provides an ideal site for bone degradation [Scott and Pease, 1956]. Ruffled borders are always circumscribed by an organelle-free area, the clear zone, which contains numerous contractile/attachment filaments arranged in association with specific integrins [Holtrop and King, 1977; Lakkakorpi et al., 1989]. It is likely that this specialized cytoplasmic region limits the acidic environment necessary for matrix degradation by sealing off the area under the ruffled border from extracellular spaces [Schenk et al., 1967; Lakkakorpi and Väänänen, 1991]. Other distinctive morphological features of active osteoclasts include nuclei with prominent nucleoli, a strongly basophilic, vacuolated cytoplasm, highly developed perinuclear Golgi complexes, numerous mitochondria, and abundant free ribosome, and lysosomes [Holtrop, 1991].

The observation that osteoclasts are usually found on mineralized surfaces in close association with marrow is both functional and developmental in nature. The former observation, that osteoclasts degrade bone matrix, was realized long ago; however, the origin of osteoclasts eluded researchers until recently. Among the first evidence for a hemopoietic origin of osteoclasts was the work of Donald Walker [1973], in which he cured mice of osteopetrosis by temporary parabiosis with a normal sibling. This pioneering work, together with similar parabiotic experiments concerning the nature of fracture callus formation, revealed that osteoclasts are derived from circulating precursors, the foundation for the hemopoietic origin of osteoclasts [Göthlin and Ericsson, 1973].

To resorb bone, newly formed or quiescent osteoclasts must be activated by factors in the local environment either in response to systemic signals such as 1,25-dihydroxyvitamin D3 or parathyroid hormone (PTH), or local factors including matrix components, cytokines, or growth factors produced by cells of the microenvironment [Rodan and Martin, 1981]. These factors stimulate bone resorption by influencing adhesion to bone, formation of a ruffled border, release of acid hydrolases, and production of superoxide radicals [Baron et al., 1985, 1986; Key et al., 1990].

Recent investigations about the origin, regulation, and function of osteoclasts have relied primarily on the use of isolated osteoclasts or marrow/stromal cell cultures. How these data relate

**TABLE 1. Characteristics of Available Osteopetrotic Mutations**

| | Bone Resorption[a] | | | Bone | Serum[b] | | | BMT[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ocl# | RB | Quant. | Form | Ca | Pi | 1.25 | Cure | Defect[d] | Lethal? |
| Mouse[e] | | | | | | | | | | |
| op/op | D | Yes | 1/10th | 1/D | N | I | I | N | CSF-1/LE | No |
| mi/mi | N | No | 1/20th | I | D | D | I | Yes | SC/LE | No |
| oc/oc | I | No | D | I | D | D | I | No | LE | Yes |
| gl/gl | D | Yes | D | I | D | D | ? | Yes | SC | Yes |
| Rabbit[f] | | | | | | | | | | |
| os/os | D | No | D | I/D | D | D | I | No | OCL/LE | Yes |
| Rat[g] | | | | | | | | | | |
| ia/ia | I | No | 7/10th | D | N | ? | I/D | Yes | SC | No |
| op/op | D | Yes | D | ? | N | D/N | I | Yes | SC | Yes |
| tl/tl | D | ? | 1/20th | ? | N | D/N | I | No | LE(CSF-1?) | No? |
| mib/mib | D | Yes | D | N | N | N | I/D | ? | ? | No |

[a]Ocl, osteoclast; RB, ruffled border; Quant, quantity. [b]Ca, calcium; Pi, phosphate; 1,25, vitamin D. [c]BMT, Bone marrow transplant. [d]CSF-1, colony stimulating factor-1; LE, local environment; SC, stem cell; OCL, osteoclast. [e]Mouse: op/op, osteopetrotic; mi/mi, microphthalmic; oc/oc, osteosclerotic; gl/gl, grey-lethal. [f]Rat: mib/mib, microphthalmia blanc; ia/ia, incisor absent; op/op, osteopetrotic; tl/tl, toothless. [g]Rabbit: os/os, osteosclerotic.

to in vivo events is a primary concern and raises fundamental questions. What is the origin and regulation of the specific mononuclear precursors of osteoclasts? What factors determine the tissue and site-specificity of osteoclasts and their subsequent activation? What causes resorption to stop and what is the lifespan of an osteoclast? Many of these issues can be addressed by use of specific mutations or analyses of events during tooth eruption. These opportunities are described below.

## OSTEOPETROSIS: OPPORTUNITIES FOR STUDYING SYSTEMIC OSTEOCLAST DEVELOPMENT AND FUNCTION

Osteopetrosis is a rare metabolic bone disease in mammals characterized by a general skeletal sclerosis caused by reduced bone resorption [Osier and Marks, 1992]. Because these mutations interrupt the development or function of osteoclasts, they represent reproducible models in which to study the systemic and local factors essential for osteoclast differentiation and function [Marks and Walker, 1976]. In addition, because osteoclasts fail to develop in certain skeletal sites in different osteopetrotic mutations, these mutants offer models in which to explore the emerging site-specificities of osteoclast development and function [Marks et al., 1993]. The laboratory animals with osteopetrosis which have been useful in the study of osteoclast development and function are listed with their distinguishing characteristics in Table I.

The general skeletal manifestations of osteopetrosis are remarkably similar. Because the disease is invariably caused by reduced bone resorption, any process dependent on bone resorption is compromised. All mutants have a generalized sclerosis detectable radiographically at birth due to absent or delayed marrow cavity formation. The ends of long bones lack the external modeling present in normal animals and delay or failure of tooth eruption is common among the osteopetroses.

Despite these similarities at the tissue level, the osteopetroses exhibit a remarkable heterogeneity with respect to osteoclast morphology and function [Marks, 1987; Seifert et al., 1993]. While osteoclast activity is reduced in all mutations, and osteoclast numbers are reduced in most, the numbers of osteoclasts per unit area of the skeleton are elevated in the *ia* rat and *oc* mouse [Marks et al., 1984]. Ruffled borders are absent or poorly developed in most mutations, but those few osteoclasts in the skeleton of *op* mice have extensive ruffled borders [Marks, 1982].

In addition to the morphological and functional aberrations present in osteoclasts in osteopetrotic animals, osteoblast abnormalities in three rat mutations have recently been reported [Shaloub et al., 1991], and bone formation is increased in the four mouse mutations (*gl, oc,*

*op,* and *oc*) but decreased in both the *ia* rat and *os* rabbit [Marks, 1989]. The abnormal numbers and/or function of osteoblasts in *tl* rats and *os* rabbits, together with the alterations in osteoblastic gene expression among the rat mutations, suggest that derangements in osteoblasts may contribute and/or compound abnormal osteoclast function in osteopetrotic animals. This is particularly significant because the reported derangements in gene expression are associated with functional properties of the osteoblast, including some believed to be involved in osteoclast recruitment and/or development.

Additional morphological and physiologic evidence for alterations in the skeletal microenvironment of osteopetrotic mutants includes, in *oc* mice [Seifert and Marks, 1985], an amorphous material between the calcified cartilage and newly formed bone which can not be resorbed by either mutant or normal osteoclasts [Van Slyke and Marks, 1987], and impaired production of colony-stimulating factor-1 (CSF-1 or M-CSF) in the *op* mouse mutation [Wiktor-Jedrzejczak et al., 1990; Felix et al., 1990; Yoshida et al., 1990]. The relationship of these findings to the osteoclast biology in these mutations are being explored.

Extraskeletal manifestations of congenital osteopetrosis in animals include elevations in serum 1,25-dihydroxyvitamin $D_3$(1,25$(OH)_2$D) at some point in life, hypocalcemia, and hypophosphatemia (see Table I). These data suggest that some osteopetrotic individuals possess a selective skeletal resistance to 1,25$(OH)_2$D [Marx and Barsony, 1988], a phenomenon often paralleled by advanced development of the intestinal vitamin D-dependent calcium binding protein calbindin-$D_{9k}$ [Seifert et al., 1988]. In addition, mRNA for the intestinal 1,25$(OH)_2$D receptor (VDR) is abnormal in some mutations, suggesting that the increased intestinal VDR in these rats is a compensatory mechanism, increasing intestinal calcium absorption to maintain normal serum calcium levels [Popoff et al., 1992]. Studies of the regulation of VDR by PTH [Reinhardt and Horst, 1990], together with ligand binding studies in these mutations, should provide insights on the interdependency of bone and vitamin D.

Additional evidence for 1,25$(OH)_2$D abnormalities has come from the renal studies of Nesbitt and others [1992], who reported elevated levels of renal 25-hydroxyvitamin D-1-alpha-hydroxylase in *oc, mi,* and *op* mice. This enzyme produces the active metabolite of vitamin D, suggesting that increased synthesis is the major cause of high levels of circulating 1,25$(OH)_2$D in these mutants. These data, together with elevated serum levels of 1,25$(OH)_2$D in osteopetrotic animals suggest that skeletal resistance to 1,25$(OH)_2$D is characteristic of congenital osteopetrosis in mammals [Seifert et al., 1993]. Clearly, the relationship between the skeleton and endocrine systems is complex, and deserves further study.

Some osteopetrotic mutations are cured by stem cell replacement (see Table I) and include the *op* and *ia* rats and the *mi* and *gl* mice. These findings were the result of the pioneering work of Walker [1973], which led to the first successful clinical treatment of human infantile osteopetrosis [Coccia et al., 1980; Sorell et al., 1981]. However, stem cell therapy does not cure osteopetrosis in *op* or *oc* mice, *tl* rats, or *os* rabbits indicating that the defect in these mutations is not in the stem cells themselves but in other factors, including the skeletal microenvironment [Seifert et al., 1993].

Experimental investigations of osteopetrotic mutations have contributed to our understanding of bone cell biology, especially osteoclast development and function [Marks, 1989]. These contributions include advances in understanding calcium and phosphate homeostasis, the regulation of bone resorption, and the origin and function of osteoclasts. These animal models are likely to provide additional insights concerning bone cell biology, including the role of CSF-1 and other factors in the regulation of generalized and site-specific osteoclastogenesis, end organ resistance to vitamin D, control of bone modeling and remodeling by local factors and cells, and clinical management of the transplant-resistant osteopetroses.

## TOOTH ERUPTION: AN OPPORTUNITY TO STUDY LOCALIZED OSTEOCLAST DEVELOPMENT AND FUNCTION

Tooth eruption is defined as the dynamic process whereby a tooth is translocated from its developmental site in alveolar bone to its functional position in the oral cavity [Massler and Schour, 1941]. During this process, bone above the erupting tooth is removed while new bone forms below it. These events represent a localized, bilaterally symmetrical, and precisely timed biological growth process [Cahill et al., 1988]. The precise local timing of the cellular, biochemi-

cal, and molecular events which cause tooth eruption, and the necessity of bone resorption for eruption, provide a unique opportunity for the study of local osteoclast development and function.

Several lines of evidence show that bone resorption is a necessary and rate-limiting requirement for eruption, and that the dental follicle, a thin vascular dense connective tissue investment of the developing tooth germ, directs resorption and eruption. First, the radiographic and histologic hallmark of bone resorption during tooth eruption is the timely formation of an eruption pathway [Marks et al., in press]. Second, teeth do not erupt when bone resorption is absent or reduced. This is best illustrated in the spectrum of aberrant tooth eruption observed among the mammalian osteopetroses. Teeth do not erupt in the most severe forms of the disease (e.g., *tl,* toothless rat), while absence of erupted incisors and delays and/or absence of erupted molars is observed in mutations with less severe reductions in bone resorption (*mib,* microphthalmia blanc, *ia,* incisors-absent rats) [Seifert et al., 1993]. Earlier erupting teeth are most affected in these mild forms. Third, tooth eruption is restored in osteopetrotic animals with treatments that increase bone resorption, such as stem cell replacement and administration of CSF-1 [Marks, 1981; Iizuka et al., 1992]. Fourth, tooth eruption is blocked by surgical removal of the dental follicle or clinical procedures which damage the follicle, which prevents bone resorption and formation of an eruption pathway [Cahill and Marks, 1980; Barfoed et al., 1984]. Fifth, blocking bone resorption by local delivery of bafilomycin $A_1$, an inhibitor of osteoclastic proton pumps, halts tooth eruption [Sundquist and Marks, in press]. Sixth, eruption and bone resorption are independent of the tooth. Cahill [1969] demonstrated that the formation of an eruption pathway occurs on schedule even in surgically impacted teeth, and metal replicas of dog premolars erupt on schedule provided they are surgically placed within the corresponding dental follicle after removal of the tooth [Marks and Cahill, 1984].

Scanning electron microscopy of mineralized surfaces of the bony crypts surrounding erupting teeth demonstrates that the crypt consists of three different topographical regions: a coronal area characteristic of bone resorption, an apical region characteristic of forming bone, and an intermediate zone typical of resting bone undergoing neither resorption nor formation [Marks et al., in press]. Light and electron microscopy of these surfaces reveal that they are covered with the appropriate cellular correlates which include numerous active osteoclasts and osteoblasts on coronal and apical crypt surfaces, respectively [Marks et al., 1983; Marks and Cahill, 1986]. These observations imply that the dental follicle directs tooth eruption by coordinating bone resorption and formation on opposite sides of an erupting tooth.

The mechanisms by which the follicle regulates these processes are currently being examined. Tooth eruption is preceded by a cellular infiltration of mononuclear cells into the coronal part of the dental follicle in rats and dogs. These cells are potentially the precursors of the osteoclasts which form the eruption pathway, since they have ultrastructural and histochemical features of preosteoclasts and their arrival in the follicle coincides with the appearance of osteoclasts on adjacent alveolar bone surfaces [Marks et al., 1983; Marks and Grolman, 1987; Wise and Fan, 1989]. Recent studies have shown that daily injections of colony-stimulating factor-1 (CSF-1) accelerate the appearance and increase the number of these two cell populations around erupting rat molars, promoting tooth eruption in the toothless (osteopetrotic) rat, and accelerating eruption in normal animals [Iizuka et al., 1992; Cielinski et al., in press].

Biochemical analyses of the dental follicle of canine permanent premolars have provided additional evidence for the role of the dental follicle in tooth eruption. During eruption, collagen and proteoglycan content increase 250% and 45%, respectively [Gorski et al., 1988]. The dental follicle contains more than 20 proteins prior to eruption, including a sialoprotein of 95,000 relative molecular weight (DF-95) which is reduced at the onset of eruption, when three new lower molecular weight sialoproteins appear. Since the relative abundance of DF-95 and the three new sialoproteins present at the onset of eruption equal the total content of DF-95 prior to eruption, the fragmentation of DF-95 is a biochemical marker for the beginning of eruption [Gorski et al., 1988]. Additional studies have shown that the dental follicle contains collagenolytic activity [Woessner and Cahill, 1974] and the amount of metalloproteinases (collagenase and stromelysin) is reduced during eruption [Gorski and Marks, 1992], suggesting a role for these enzymes in tooth eruption. Recently, Gorski and

others [in press] demonstrated that DF-95 is localized to the reduced enamel epithelium, providing biochemical evidence that this derivative of the oral epithelium is involved in eruption. Numerous proteases have been identified in the enamel organ during tooth development [Nanci et al., 1989], and their activation at the completion of crown formation may contribute to the eruption process by fragmenting DF-95 and initiating the release of metalloproteinases from the follicle.

Our understanding of the molecular regulation of tooth eruption is in its infancy. Cohen [1962] demonstrated that injections of epidermal growth factor (EGF) cause precocious eruption of incisors in rodents. Recently, EGF and EGF-receptor have been localized to cells of the dental follicle and enamel organ early in erupting rat molars [Wise et al., 1992], EGF increases interleukin-1 (IL-1) in enamel organ cultures, and IL-1 increases CSF-1 in dental follicle cells in vitro [Wise and Lin, in press]. In addition, cells of the dental follicle produce mRNA for CSF-1 and secrete the protein in culture [Wise et al., in press]. These data, combined with the observation that EGF upregulates the production of CSF-1 message and protein [Abboud, 1992], and the aforementioned data concerning the effects of CSF-1 on osteoclastogenesis suggest that both of these growth factors are involved in the molecular cascade which produces the bone resorption of tooth eruption (Fig. 1). The precise local timing of these cellular and molecular events illustrate that tooth eruption provides a unique opportunity to study the local regulation of osteoclast ontogeny and function. Furthermore, the presence of bone resorption and formation around erupting teeth provide a unique setting in which to explore their coordination in a single location.

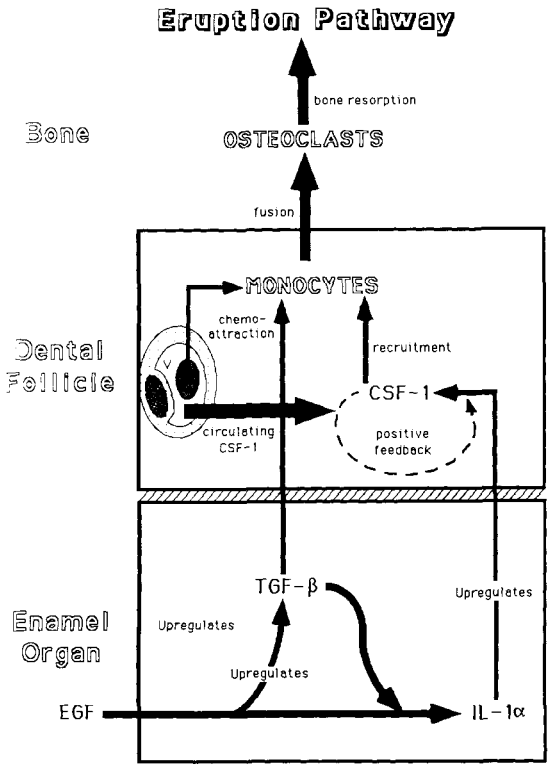


**Fig. 1.** Diagrammatic representation of a hypothesis about the molecular regulation of the bone resorption of tooth eruption. The accuracy of this hypothesis, taken largely from the cell and tissue culture data of Wise and colleagues [1992, 1994], is currently being examined in vivo.

## ACKNOWLEDGMENTS

This work was supported by grants DE07444, DE08952, DE05996, and AR39737. We thank Gary E. Wise and Jeffrey P. Gorski for helpful discussions and suggestions, and Cindy Richards for typing the manuscript.

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